Supporting information for

A Two-photon Fluorescence Turn-on Probe for Nitroxyl (HNO) and Its Bioimaging Application in Living Tissues

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Materials and instrumentation

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer. NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard. Electronic absorption spectra were obtained on a Labtech UV Power PC spectrometer. Photoluminescent spectra were recorded at room temperature with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; The fluorescence images were acquired with an Olympus FV1000 equipped with a CCD camera; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Qingdao Ocean Chemicals.

General procedure for the spectra measurement

The stock solution of the probe **GCTPOC-1** was prepared at 0.5 mM in EtOH. The solutions of various testing species were prepared from Na_2S, $FeCl_3$, NaN_3, $NaNO_2$, $NaNO_3$, Sodium ascorbate, GSH, cysteine, H_2O_2, NaClO in the twice-distilled water, and Superoxide ($O_2^{\cdot -}$) and CORM-2 (CO) in the DMSO. AS (the HNO source) and DEA/NONOate (the NO source) in 0.01M NaOH. The test solution of the probe **GCTPOC-1** (5.0 μM) in 3 mL 25 mM PBS buffer (pH 7.4) with 1% ethanol was prepared by placing 0.03 mL of the probe **GCTPOC-1** stock solution and 0.03 mL ethanol in 2.97 mL of the aqueous buffer. The resulting solution was shaken well and incubated with appropriate testing species for 45 min at ambient temperature before recording the spectra. Unless otherwise noted, for all measurements, the excitation wavelength was 410 nm, the excitation slit widths were 5 nm, and emission slit widths were 5 nm.

Experiment section:

Synthesis of the probe GCTPOC-1

Compound **GCTPOC** (20.0 mg, 0.09 mmol), 2-(diphenylphosphino) benzoic acid (34.1 mg, 0.11 mmol), DMAP (83 mg, 0.68 mmol) and DCC (24.8 mg, 0.12 mmol) were dissolved in anhydrous CH_2Cl_2 (3 mL), and the reaction mixture was stirred at room temperature for 8 hours under N_2 atmosphere. Then the mixture was concentrated under vacuum, and the crude product was purified by silica gel column chromatography (petroleum/CH_2Cl_2= 2:3) to give the compound **GCTPOC- 1** as a yellow solid (31.7mg, 0.063 mmol, 70%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 1.65-1.74 (m, 1H) , 1.88-1.97 (q, 2H), 2.37-2.43 (m, 3H), 5.05-5.09 (m, 1H), 6.61-6.64 (m, 2H), 6.89-6.93 (m, 1H), 7.19-7.23 (m, 4H), 7.37-7.42 (m, 8H), 7.58-7.64 (m, 2H), 8.20-8.23 (q, 1H); ^{13}C NMR (125 MHz, d_6-DMSO) δ 196.5, 164.6, 156.2, 152.7, 140.5, 140.3, 137.3, 134.1, 133.9, 133.7, 133.3, 131.5, 130.7, 129.3, 129.0, 120.2, 115.8, 109.8, 74.6, 38.6, 29.1, 17.4; ^{31}P NMR(d_6-DMSO) δ -5.64; MS (ESI) m/z 543.1 $[M+K]^+$. HRMS (EI) m/z calcd for $C_{32}H_{25}O_4P$: 504.1471; Found 504.1485, Anal. calcd for $C_{32}H_{25}O_4P$: C, 76.18; H, 4.99. found: C, 76.31; H, 4.67.

Conversion of GCTPOC-1 to GCTPOC

The compound **GCTPOC-1** (20 mg, 0.040 mmol) was dissolved in anhydrous EtOH (7 mL) and PBS (5 mL), and then AS (5.3 mg, 0.043 mmol, in 2 mL 0.01 M NaOH) was added, The resulting solution was stirred at room temperature for 3 h. Subsequently, the reaction mixture was adjusted to acid pH with 1N HCl, and then extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated. Purification by silica gel column chromatography (CH_2Cl_2/ ethanol = 100: 0 to 50 : 1) afforded 3.2 mg (0.015 mmol, 40%) of the compound **GCTPOC** as a yellow solid: ^{1}H NMR (400 MHz, d_6-DMSO) δ 1.64-1.71 (q, 1H) , 1.84-1.95 (m, 2H), 2.35 (d, J=8 Hz, 3H), 4.95 (d, J=8 Hz, 1H), 6.29 (s, 1H), 6.42 (d, J = 8Hz, 1H), 7.23 (d, J = 8Hz, 1H), 7.34 (s, 1H), 10.17 (s, 1H); MS (ESI) m/z 216.3 $[M]^+$.

Cell culture and fluorescence imaging

HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were plated in 35 mm glass-bottom culture dishes and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. The cells were further incubated with **GCTPOC-1** (5 μM) for 20 min at 37 °C, After washing with PBS three times to remove the remaining **GCTPOC-1**, the HeLa cells were incubated in the absence or presence of AS (35 μM or 75 μM) in the culture medium for 45 min at 37 °C, and imaged with an Olympus FV1000 equipped with a CCD camera.

Preparation of fresh mouse liver slices and two-photon fluorescence imaging.

Slices were prepared from the liver of 14-day-old mice. Slices were cut to 400 μm thickness by using a vibrating-blade microtome in 25 mM PBS (pH 7.4). For the control experiments, slices were incubated with 30 μM **GCTPOC-1** in PBS buffer bubbled with 95% O_2 and 5% CO_2 for 1 h at 37 ˚C. Slices were then washed three times with PBS, transferred to glass-bottomed dishes, and observed under a two-photon confocal microscope (Olympus FV1000). To obtain the two-photon fluorescence images of the tissues incubated with both the probe and AS, the slices were pre-treated with 30 μM **GCTPOC-1** for 40 min before the AS was added. Following this incubation for 1 h at 37 °C, the slices were washed three times and imaged. The two-photon fluorescence emission was collected at between 520 and 570 nm upon excitation at 780 nm with a femtosecond pulse.

Determination of the fluorescence quantum yield: [1] Fluorescence quantum yields for **GCTPOC-1** and **GCTPOC** were determined by using Rhodamine 6G (Φ_F =0.95 in water) as a fluorescence standard.[1] The quantum yield was calculated using the following equation:

$$\Phi_{F(X)} = \Phi_{F(S)} \, (A_S F_X / A_X F_S) \, (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, and n is the

refractive index of the solvent used. Subscripts S and X refer to the standard and to the unknown, respectively.

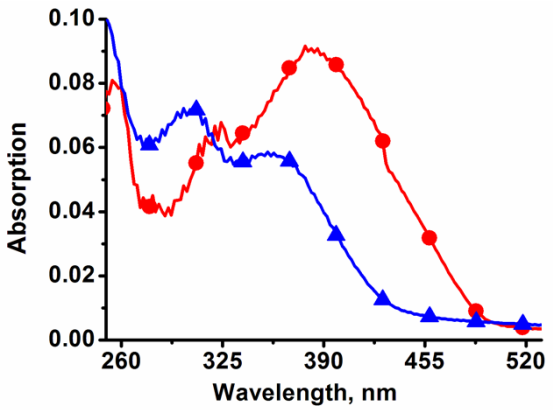

Fig. S1 Absorption spectra of the probe **GCTPOC-1** (10 μM, ▲) and the reference compound **GCTPOC** (10 μM, ●) in PBS buffer (25 mM, pH 7.4, 1% EtOH).

Detection limit: The detection limit was determined from the fluorescence titration data based on a reported method.[2] According to the result of titration experiment, the fluorescent intensity data at 512 nm were normalized between the minimum intensity and the maximum intensity. A linear regression curve was then fitted to these normalized fluorescent intensity data and the point at which this line crossed the axis was considered as the detection limit (5.90×10^{-7} M).

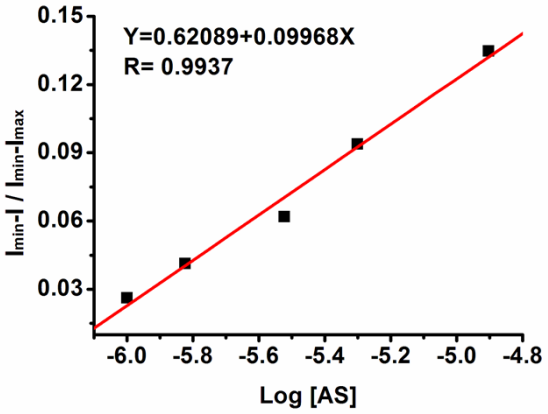

Fig. S2 Normalized response of fluorescence signal by changing the concentration of AS.

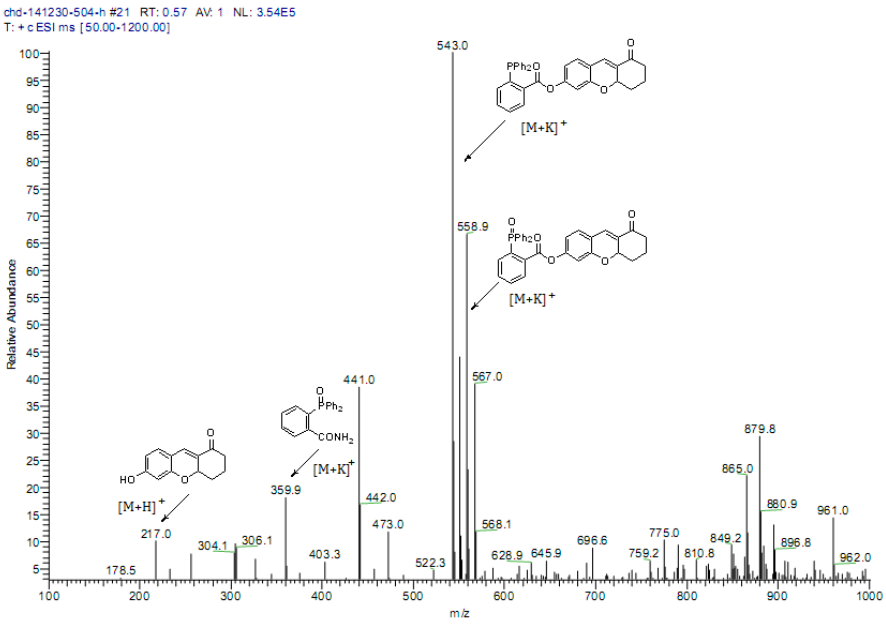

Figure S3. ESI-MS spectrum of **GCTPOC-1** (1 mL **GCTPOC-1** at 250 μM, EtOH/PBS=1:1) with AS (0.5 mL AS at 2 mM, 0.01M NaOH).

Kenetic studies: The reaction of **GCTPOC-1** (5 μM) with AS in PBS (25 mM, pH 7.4, 1% EtOH) was monitored using the fluorescence intensity at 512 nm. The reaction was carried out at 37 ˚C. The *pseudo*-first-order rate constant for the reaction was determined by fitting the fluorescence intensities of the samples to the *pseudo*-first-order equation:

$$\text{Ln}\,[(F_{max} - F_t) / F_{max}] = - k't$$

Where F_{max} and F_t are the fluorescence intensities at 512 nm at time t and the maximum value obtained after the reaction was complete. *k'* is the *pseudo*-first-order rate constant. The *pseudo*-first-order plots for the reaction of **GCTPOC-1** with 15 equiv. of AS is shown in Figure S4, The negative slope of the line provides the *pseudo*-first-order rate constant for HNO.

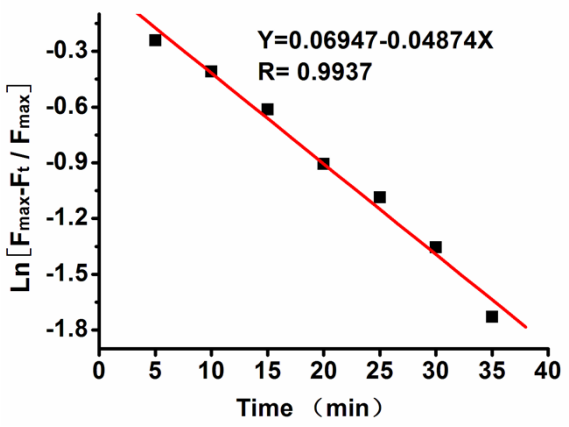

Fig. S4 *Pseudo*-first-order kinetic plot of the reaction of **GCTPOC-1** (5 μM) with AS (15 equiv) in PBS (25 mM, pH 7.4, 1% EtOH). Slope = 0.0487 min^{-1}.

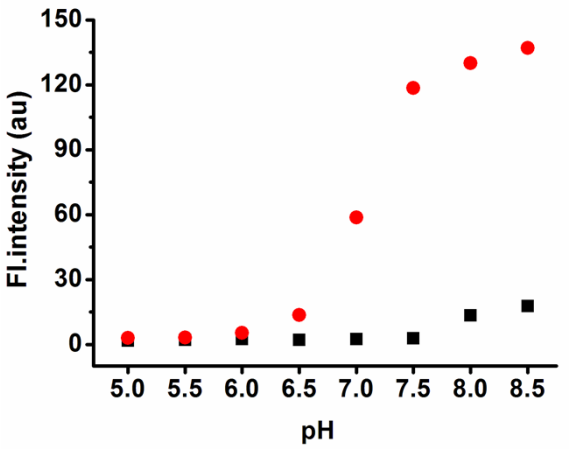

Fig. S5 Fluorescence intensity changes of the probe **GCTPOC-1** (5.0 μM) at different pH values in the absence (■) or presence (●) of AS (15 equiv).

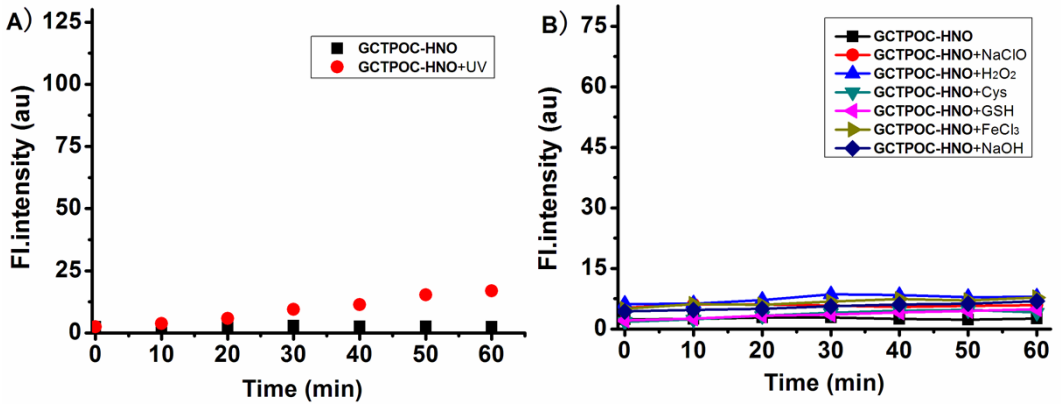

Fig. S6 (A) Photostability profiles of **GCTPOC-1** (5.0 μM) in the absence [■] or presence of UV-irradiated (●) (365 nm). The fluorescence intensities at 512 nm were continuously monitored at time intervals in PBS (25 mM, pH 7.4, 1% EtOH). Time points represent 0, 10, 20, 30, 40, 50, and 60 min. (B) Chemical stability profiles of **GCTPOC-1** (5.0 μM) in the absence [■] or presence of alkali, oxidizing and reducing reagents: blank (■), NaClO (100 μM, ●), H_2O_2 (100 μM, ▲), Cys (100 μM, ▼), GSH (1 mM, ◀), Fe^{3+} (100 μM, ▶), NaOH (0.75 mM, ♦). The fluorescence intensities at 512 nm were continuously monitored at time intervals in PBS (25 mM, pH 7.4, 1% EtOH). Time points represent 0, 10, 20, 30, 40, 50, and 60 min.

Cytotoxicity assays:

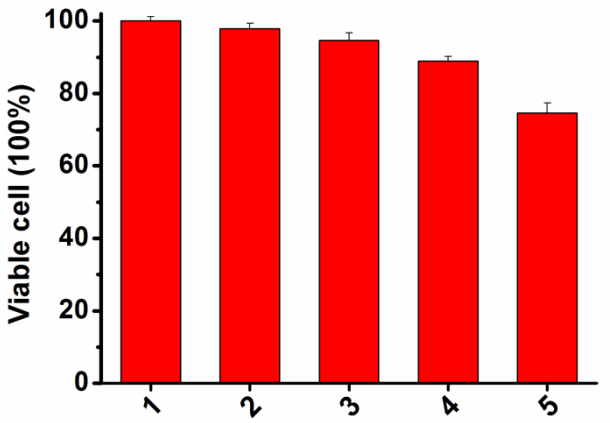

Fig. S7 Cytotoxicity assay of **GCTPOC-1** at different concentrations (1: 0 μM; 2: 5 μM; 3: 10 μM; 4: 30 μM; 5: 50 μM) for HeLa cells.

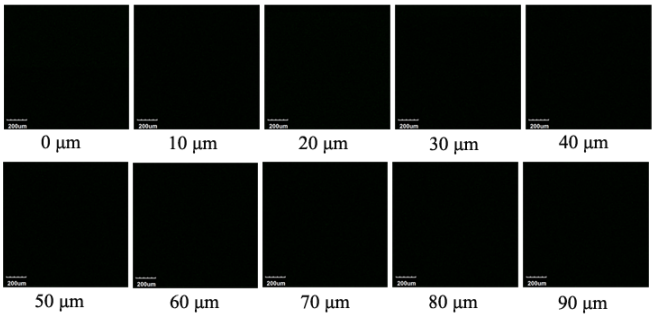

Fig. S8 One-photon fluorescence images of a fresh rat liver slice incubated with 30 μM **GCTPOC-1** *in the absence of* AS at the depths of approximately 0~90 μm. Excitation at 405 nm, Scale bar = 200 μm.

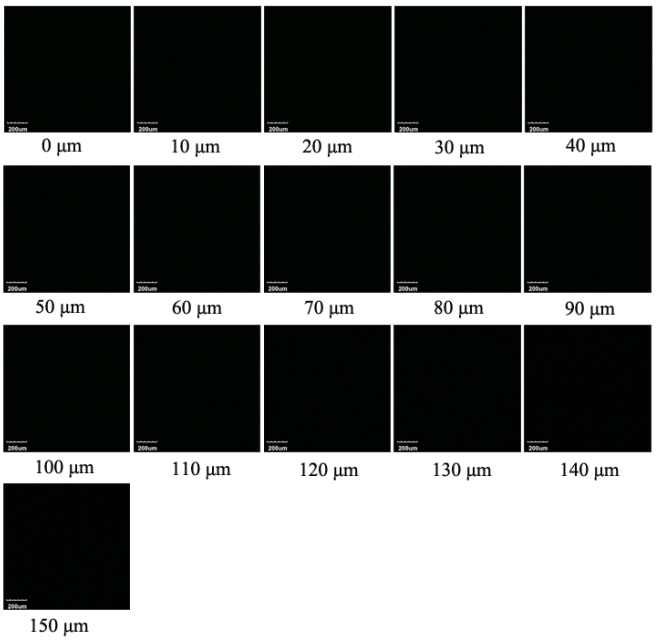

Fig. S9 Two-photon fluorescence images of a fresh rat liver slice incubated with 30 μM **GCTPOC-1** *in the absence of* AS at the depths of approximately 0~150 μm. Excitation at 780 nm, Scale bar = 200 μm.

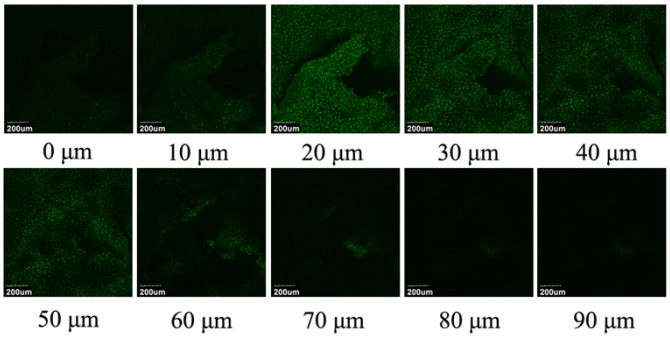

Fig. S10 One-photon fluorescence images of a fresh rat liver slice pretreated with 30 μM **GCTPOC-1** and then with AS (1 mM) incubated at the depths of approximately 0~90 μm with a magnification at 10×. Excitation at 405 nm. Scale bar = 200 μm.

References

1 (a) D. Magde, G.. E. Rojas, P. Seybold, Photochem. Photobiol. 1999, 70, 737.
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2 (a) M. Shortreed, R. Kopelman, M. Kuhn, B. Hoyland, Anal. Chem., 1996, 68, 1414; (b) A. Caballero, R. Martınez, V. Lloveras, I. Ratera, J. Vidal-Gancedo, K. Wurst, A. Tarraga, P. Molina, J. Veciana, J. Am. Chem. Soc., 2005, 127, 15666.
(c) W. Lin, L. Yuan, Z. Cao, Y. Feng, L. Long, Chem. Eur. J., 2009, 15, 5096.

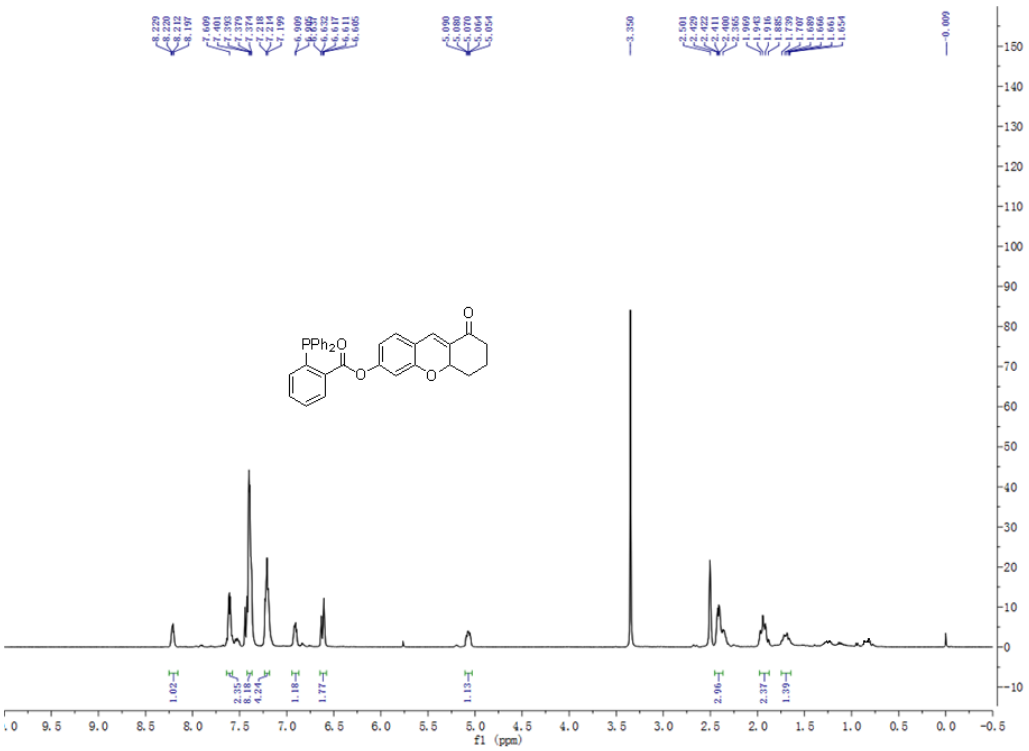

Fig. S11. ^{1}H NMR spectrum of the probe **GCTPOC-1**.

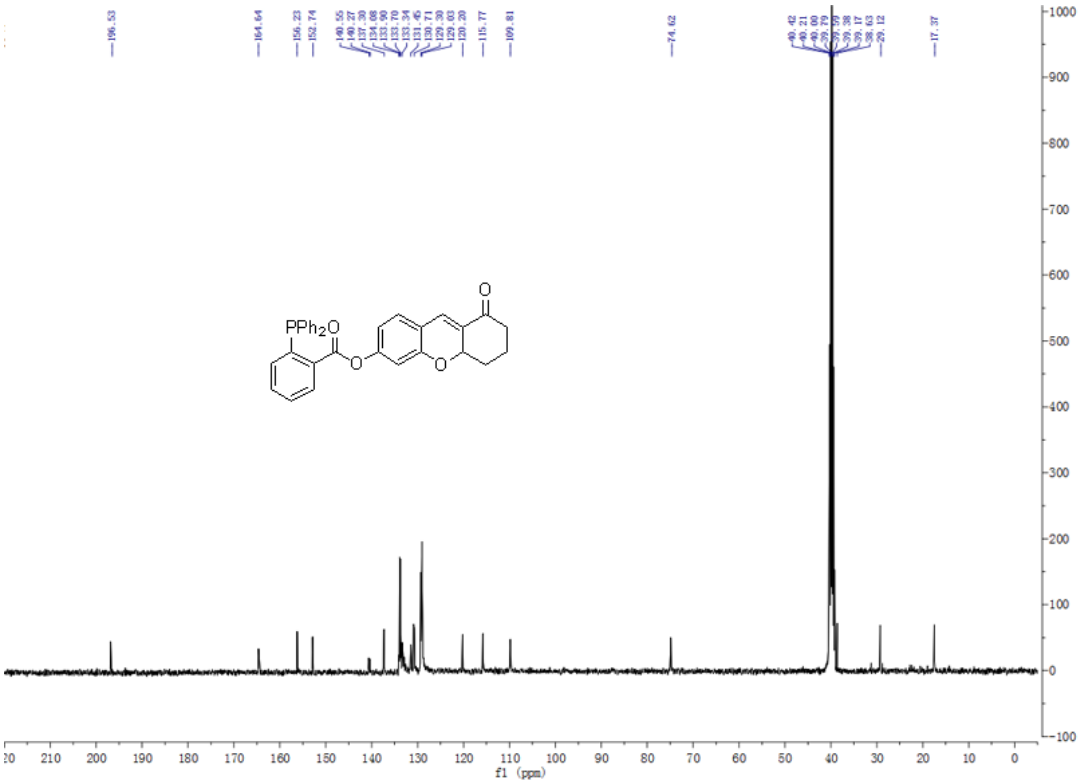

Fig. S12 ^{13}C NMR spectrum of the probe **GCTPOC-1.**

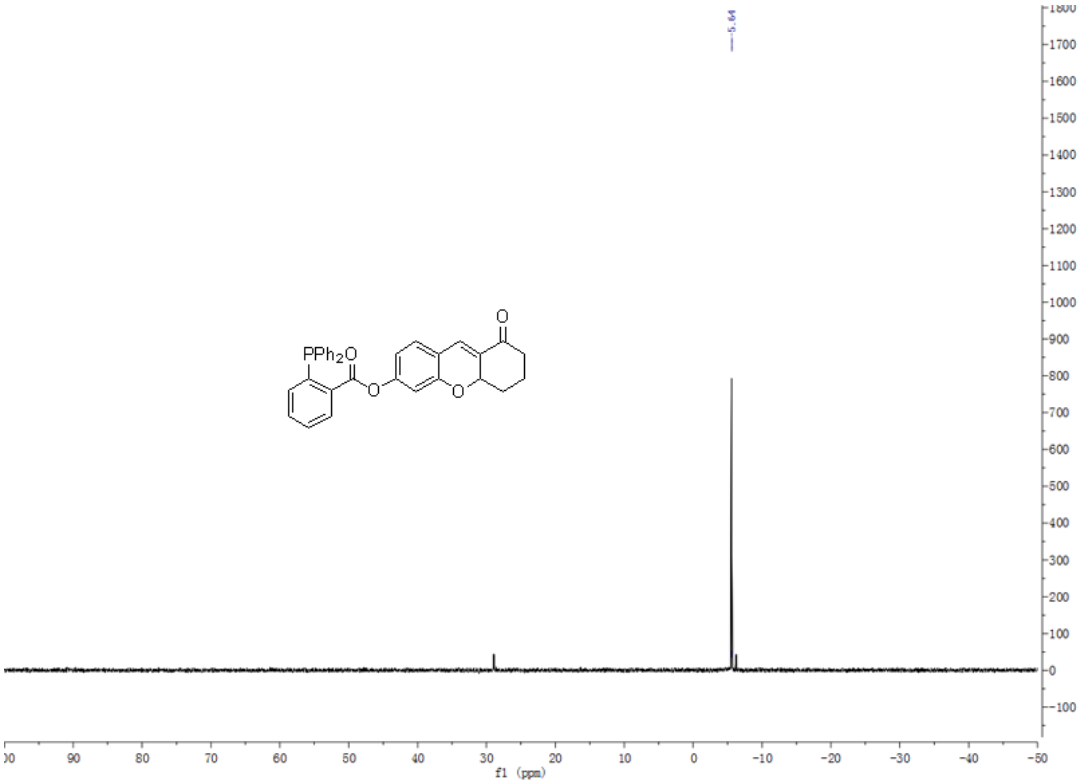

Fig. S13 ^{31}P NMR spectrum of the probe **GCTPOC-1.**

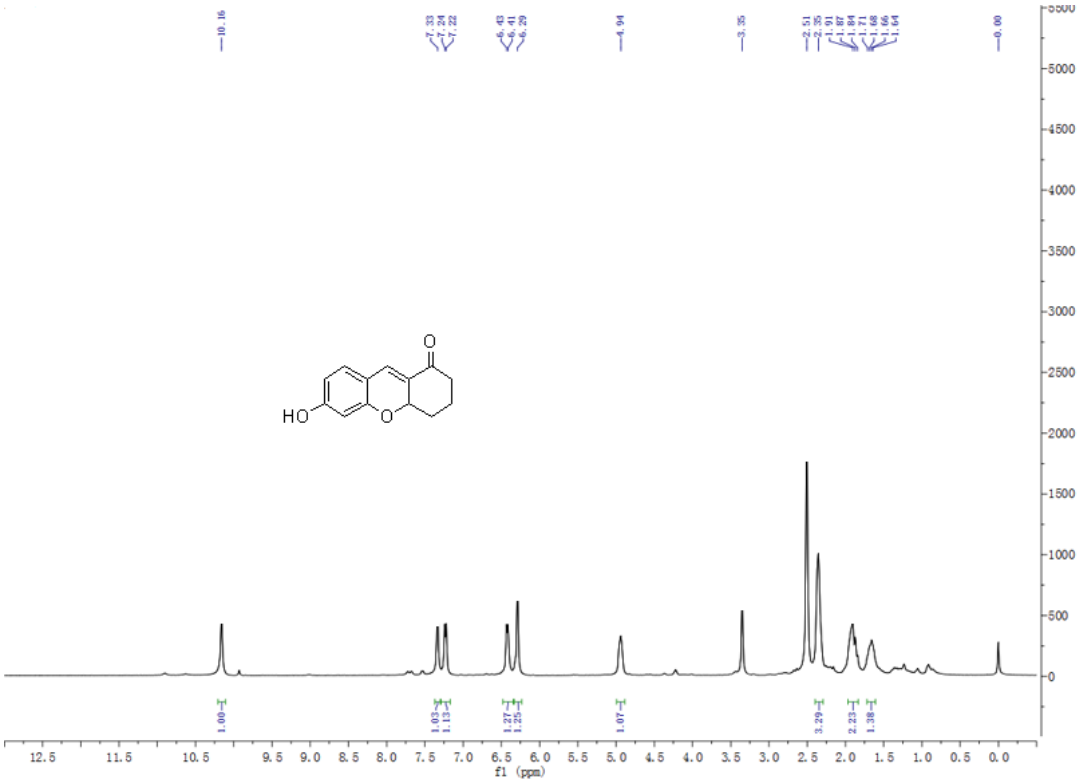

Fig. S14 ^{1}H NMR spectrum of the compound **GCTPOC**.

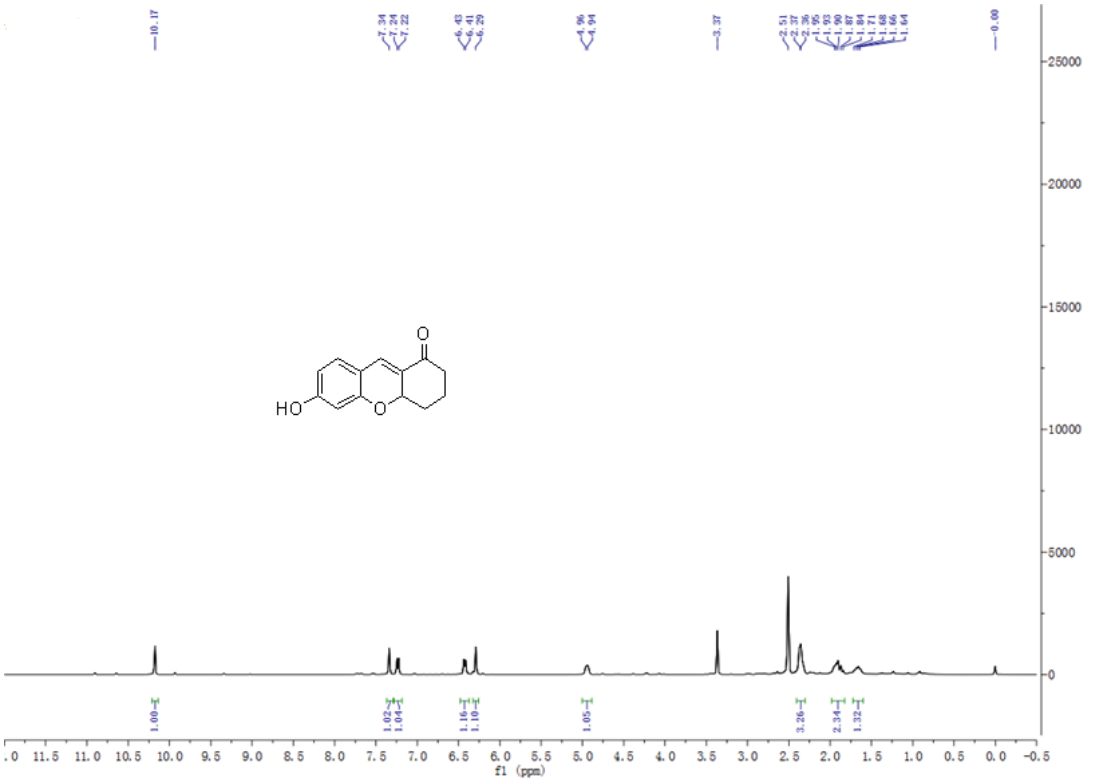

Fig. S15 ^{1}H NMR spectrum of isolated product of probe **GCTPOC-1** reacted with AS.